Brief Definitive Report

Rare Occurrence of Classical Hodgkin's Disease as a T Cell Lymphoma

By Markus Müschen,*‡ Klaus Rajewsky,* Andreas Bräuninger,§ Audrey Sylvia Baur,§‖ Joost J. Oudejans,¶ Axel Roers,* Martin-Leo Hansmann,§ and Ralf Küppers*‡

*From the *Institute for Genetics, Department of Immunology, and the ‡Department of Internal Medicine, Universität zu Köln, 50931 Köln, Germany; the §Department of Pathology, University of Frankfurt, 60596 Frankfurt, Germany; the ‖University Institute of Pathology, University Hospital CHUV, 1011 Lausanne, Switzerland; and the ¶Department of Pathology, Free University Hospital, 1081 HV Amsterdam, The Netherlands*

Abstract

Recent work identified Hodgkin and Reed-Sternberg (H/RS) cells in classical Hodgkin's disease (cHD) as clonal progeny of mature B cells. Therefore, it is generally assumed that cHD homogenously represents a B cell lymphoma. In a subset of cHD, however, H/RS cells expressing T cell–associated proteins may be candidates for alternative lineage derivation. Single H/RS cells with cytotoxic T cell phenotype were micromanipulated from three cases of cHD and analyzed by single cell polymerase chain reaction for immunoglobulin heavy (IgH) and light chain (IgL) gene rearrangements, T cell receptor (TCR)-β gene rearrangements, and germline configuration of the IgH and TCR-β loci. H/RS cells from two cases of cHD harbored clonal, somatically mutated Ig gene rearrangements, whereas TCR-β loci were in germline configuration. In contrast, H/RS cells from an additional case harbored clonal TCR-β variable/diversity/joining (VDJ) and DJ gene rearrangements, whereas the IgH locus was in germline configuration on both alleles. Thus, in two cases of cHD with H/RS cells expressing cytotoxic T cell molecules, the tumor cells are derived from mature B cells that aberrantly express T cell markers. In a third case, however, H/RS cells were derived from a T cell, demonstrating that cHD can also occur as a T cell lymphoma.

Key words: Hodgkin's disease • T cell receptor genes • immunoglobulin genes • somatic hypermutation • Epstein-Barr virus

Introduction

In classical Hodgkin's disease (cHD), the malignant Hodgkin and Reed-Sternberg (H/RS) cells typically account for <1% of cells within a complex admixture of lymphocytes, plasma cells, histiocytes, and eosinophils (1). The origin of H/RS cells in cHD was enigmatic and a matter of debate for more than a decade. Although H/RS cells usually lack expression of B lineage markers, there is now strong evidence that H/RS cells represent the outgrowth of a dominant tumor clone derived from mature B cells (2, 3). This conclusion is based on the amplification of clonally related Ig gene rearrangements from single micromanipulated H/RS cells (4, 5). The presence and pattern of somatic mutations in the rearranged V genes identified germinal center B cells as the precursors of the tumor cells (5).

In a minority of cHD cases (~5–15%), however, H/RS cells express cytotoxic T cell markers (granzyme B, perforin, and T cell intracellular antigen 1 [TIA-1]), raising the possibility that the tumor cells in these cases might originate from T lymphocytes (6–9). To clarify whether H/RS cells in such cases are indeed derived from T lymphocytes, H/RS cells were micromanipulated and subjected to single cell PCR analysis for rearranged Ig heavy chain (IgH), Igκ, and Igλ light chain genes, TCR-β VDJ and DJ gene rearrangements, as well as IgH and TCR-β germline configuration (i.e., absence of rearrangements). Whereas IgH VDJ gene rearrangements are specific for and restricted to B lineage cells, the presence of TCR-β VDJ gene rearrangements identifies a T cell. Studying H/RS cells from three such cases, a B cell

Address correspondence to Markus Müschen, Universität zu Köln, Medizinische Klinik I, LFI E4 R705, Joseph-Stelzmann-Strasse 9, 50931 Köln, Germany. Phone: 49-221-478-4490; Fax: 49-221-478-6383; E-mail: markus.mueschen@uni-koeln.de

genotype was found in two, a genotype revealing T cell origin in one case.

Materials and Methods

Clinical data on the three cases of cHD are summarized in Table I.

Immunostaining and Micromanipulation. For immunostaining, 6–7-μm frozen tissue sections were stained using antibodies against CD30 (Fig. 1 A), CD20, LMP1, anaplastic lymphoma kinase (ALK)-1 (Dako), CD15 (Fig. 1 C; Becton Dickinson), CD3 (Ortho), TCR-α/β (T Cell Diagnostics), perforin (Neo Markers), granzyme B (Monosan), and TIA-1 (Immunotech). Stained cells were mobilized and aspirated with the help of a micropipette fixed to a hydraulic micromanipulator. Multiple cells were picked from each section. Buffer covering the sections was aspirated as negative controls for PCR analysis. For positive control of PCR, single B and T cells were either micromanipulated or sorted by flow cytometry.

Single Cell PCR. To analyze individual micromanipulated cells for IgH, Igκ, Igλ, as well as TCR-β VDJ and DJ gene rearrangements or germline configuration of the IgH and TCR-β loci, whole genome preamplification (10) was performed. Aliquots from these reactions were then subjected to two rounds of seminested PCR amplification as described previously. For analysis of the IgH and TCR-β loci, three PCR strategies were applied (Fig. 2, A and B), one of which targets IgH (Fig. 2 A, iii) or TCR-β (Fig. 2 B, iii) VDJ rearrangements, a second IgH (Fig. 2 A, ii) or TCR-β (Fig. 2 B, ii) DJ rearrangements, and a third detects germline configuration of either the IgH (Fig. 2 A, i) or the TCR-β (Fig. 2 B, i) locus. Rearranged V_H, Vκ, and Vλ genes were amplified using family-specific leader or framework region V gene primers and two sets of J_H, Jκ, and Jλ primers in a seminested approach (5, 11, 12). D_HJ_H rearrangements and germline configuration within the IgH locus were detected using seven D_H family–specific primers and two sets of J_H gene–specific primers in a seminested approach (5). In the case of germline configuration of the IgH locus, a 340-bp fragment was obtained with the D_H7 primer, due to the close vicinity of the D_H7-27 gene segment and J_H1 (Fig. 2 A, i). D_H family–specific primers were as follows: 5′-GTGTGCAGGCCTCRGTCTCTGTG-3′ for the D_H1 gene family; 5′-GCACTGGGCTCAGAGTCCTCTC-3′ for the D_H2 family; 5′-CCTCAGGTCAGCCCTGGACATC-3′ for the D_H3 family; 5′-TGAGATCCCCAGGACGCAGCAC-3′ for the D_H4 family; 5′-TCCCTGGGAAGCTCCTCCTGAC-3′ for the D_H5 family; 5′-GACACCAGACAGAGGGGCAGGC-3′ for the D_H6 family; and 5′-AGAGTGACTGGCAGGGTTG-AGG-3′ for the D_H7–27 gene. Amplification of TCR-β VDJ gene rearrangements was carried out as described previously using a panel of 24 Vβ family–specific primers and two sets of Jβ gene–specific primers in a seminested approach (13). Germline configuration was detected separately for both Cβ loci (Fig. 2 B, i) using primers binding to intronic sequences flanking the Dβ1 (5′-CCCCTTCGCCAAACAGCCTTA-3′ as forward, 5′-GAG-TGAGGCAGAGGCATTCTGAAC-3′ as external reverse, and 5′-GCAGAGGCATTCTGAACCAAATTG-3′ as internal reverse primer) or the Dβ2 gene (5′-TCAGGGTGATGCATGTTC-CAAGGA-3′ as forward, 5′-GGGACCCTGCAAGACCACA-GCT-3′ as external reverse, and 5′-ACTCTTCCCACCTGGTA-GCTGCAT-3′ as internal reverse primer). DβJβ rearrangements were amplified using the primers specific for intronic sequences in the upstream regions of the Dβ1 and the Dβ2 genes, together with primers specific for the Jβ1 or Jβ2 gene clusters, respectively (Fig. 2 B, ii).

EBV infection of single H/RS cells was examined by amplification of a fragment of the EBV nuclear antigen 1 (EBNA1) gene by seminested PCR (5′-GGTCGCCGGTGTGTTCGTATATGG-3′ as forward, 5′-GCGGCAGCCCCTTCCACCATAG-3′ as external reverse, and 5′-AGGGAGGCAAATCTACTCCATCGTC-3′ as internal reverse primer).

PCR products were gel-purified and directly sequenced.

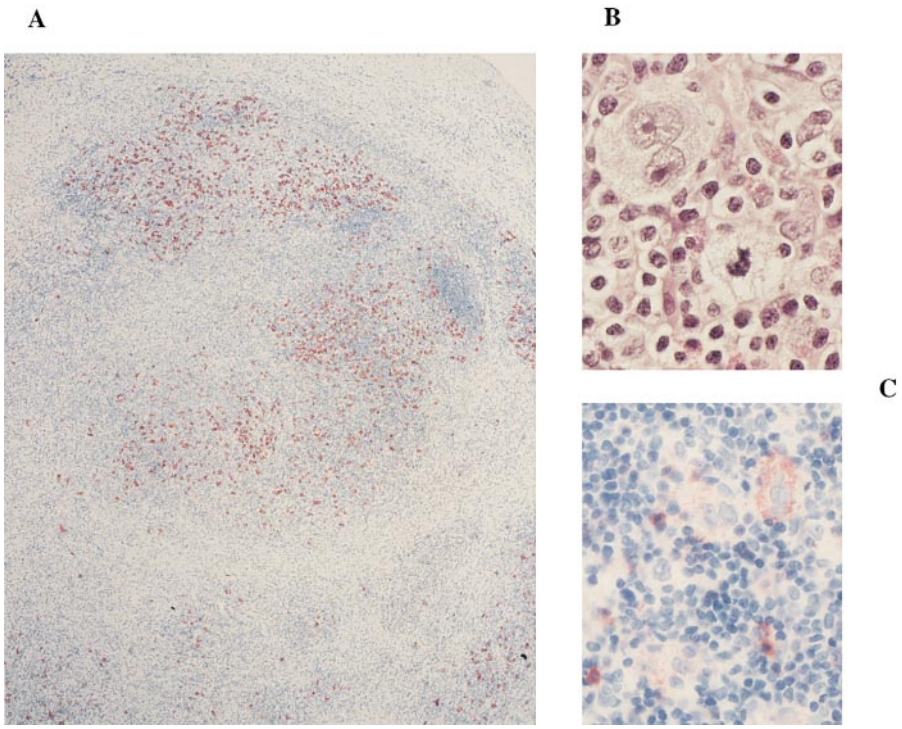

Figure 1. Immunostaining of cHD, case III. Histological stainings were as follows: (A) the tissue is stained for CD30 with the use of alkaline phosphatase (4-fold magnification); (B) hemalaun-eosin staining of some multinucleated Reed-Sternberg cells at 60-fold magnification; (C) staining for CD15 at 40-fold magnification. Staining of this case for expression of perforin is shown on the cover illustration of this issue.

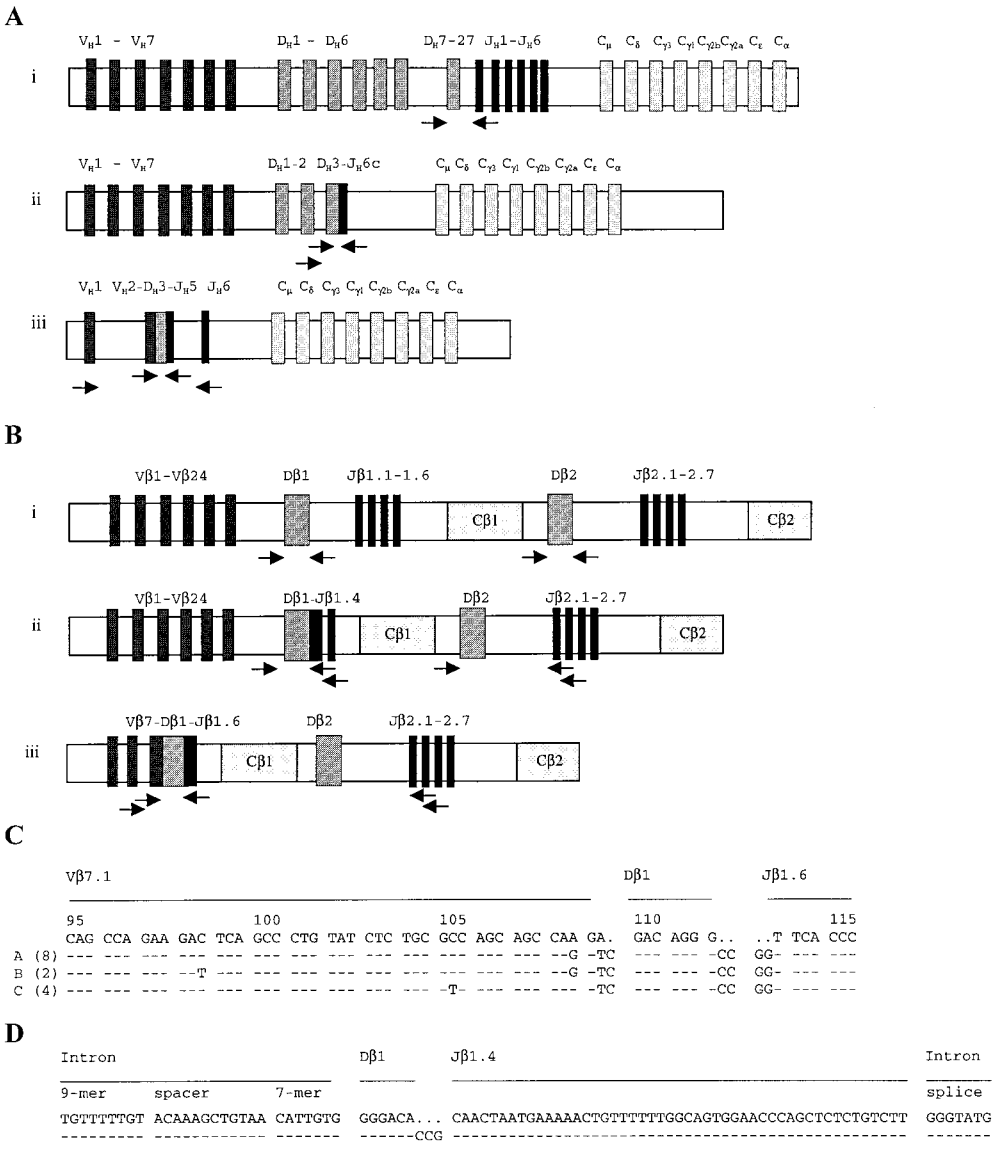

Figure 2. Amplification of IgH gene and TCR-β gene rearrangements from H/RS cells. PCR strategies for amplification of PCR products which are specific for IgH (A) or TCR-β (B) germline configuration (i), DJ gene rearrangements (ii), and VDJ gene rearrangements (iii) are depicted. V_H1-V_H7 and Vβ1-Vβ24 represent the seven Ig V_H gene families and the 24 TCR Vβ gene families; and J_H1-J_H6 and Jβ1.1-1.6 and Jβ2.1-2.7 indicate the Ig J_H and the TCR Jβ genes, respectively. The IgH DJ (A, ii) and IgH VDJ (A, iii) rearrangements and TCR-β DJ (B, ii) and TCR-β VDJ (B, iii) rearrangements depict those amplified from cases I and III, respectively. Arrows indicate the PCR primers used (not to scale). (C) A fragment (codons 95–115) of the sequence alignment of the clonal TCR Vβ7.1–Dβ1–Jβ1.6 rearrangement amplified from single H/RS cells of case III is given. The germline sequence of Vβ7.1, Dβ1, and Jβ1.6 genes (top) is compared with the clonal sequence variants (A, B, and C) obtained from eight, two, and four H/RS cells of case III, respectively. The three sequence variants (A, B, and C) differ by single nucleotide substitutions in codons 98, 105, and 108 (complete sequence data are available from GenBank/EMBL/DDBJ under accession nos. AJ243645–AJ243647). (D) Sequence alignment of the clonal TCR Dβ1–Jβ1.4 rearrangement amplified from 14 H/RS cells of case III is given. In contrast to the clonal Vβ7.1 gene rearrangement (C), the Dβ1–Jβ1.4 gene rearrangement does not exhibit intraclonal diversity (complete sequence data is available from GenBank/EMBL/DDBJ under accession no. AJ243648).

Results

H/RS cells from all three cases studied exhibit the typical immunophenotype of the tumor cells in cHD, as they coexpress CD30 and CD15 (for case III, see Fig. 1) but lack expression of the B cell antigen CD20 (Table I). H/RS cells from cases I and II express in part granzyme B (see cover of this issue), and TIA-1 and are EBV-positive, whereas H/RS cells from case III express perforin in addition to granzyme B and TIA-1 and are EBV-negative (Table I). Since the majority of anaplastic large cell lymphomas express one or more of the cytotoxic T cell markers studied here (7–9), diagnosis of cHD was further corroborated by the lack of nucleophosmin(NPM)-ALK gene expression in the H/RS cells. The NPM-ALK fusion protein arises from the translocation t(2; 5) (p23; q35), which is typically seen in anaplastic large cell lymphoma but not in cHD (14).

Efficiency of PCR amplification from single micromanipulated cells for all three cases was similar to that usually encountered (i.e., <40%, likely due to technical matters such as partial degradation or inaccessibility of DNA [3–5, 11–13]; Table II).

From case I, 50 $CD30^+$ and 15 granzyme B^+ H/RS cells were micromanipulated and analyzed by single cell PCR.

Table I. *Case Description of Patients with cHD*

Case	I	II	III
Age	31	26	51
Sex	Male	Male	Male
Presentation	First	First	First
LN biopsy site	Abdominal	Cervical	Inguinal
Stage	IIB	I	II
Hodgkin subtype	Mixed cellularity	Mixed cellularity	Nodular sclerosis
Phenotype of H/RS cells			
CD30	+	+	+
CD15	+	+	+
CD20	−	−	−
CD3	−	−	−
TCR-α/β	−	ND	−
Granzyme B	+ (90%)	+ (60%)	+ (40%)
Perforin	−	−	+ (100%)
TIA-1	+ (60%)	+ (>90%)	+ (40%)
EBV	+	+	−
ALK	−	ND	−

Numbers in parentheses indicate percentages of positive Reed-Sternberg cells. Presence or absence of EBV was determined by amplification of a fragment of the EBNA1 gene from single H/RS cells, and the results were further confirmed in case I by LMP1 staining and in case III by EBER in situ hybridization. ALK expression is indicative for the t(2;5) translocation resulting in the expression of a fusion molecule with NPM.

From multiple H/RS cells of both subsets, a clonal Ig V_H2-5–D_H3-10–J_H5b and a clonal D_H3-9–J_H6c gene rearrangement were amplified. The V_H2 gene was rearranged in-frame, all sequences were identical, and the rearrangement was rendered nonfunctional by a somatic mutation generating a translation stop in codon 91 of framework region III (Table III). Thus, case I represents another example in which the H/RS cells have lost their capacity to express antigen receptor due to deleterious somatic mutations (5). No IgL, TCR-β VDJ, or DJ gene rearrangements were obtained analyzing multiple cells (Table II). Fragments corresponding to germline configuration of the IgH locus were not obtained, but fragments specific for germline configuration of the TCR Cβ1 and Cβ2 loci were repeatedly amplified (Table II). For the TCR Cβ2 locus, germline configuration could be assigned to both alleles because of the detection of two polymorphic forms of the Dβ2 gene (G and/or A at position 13 of the Dβ2 gene; see reference 15).

Case II, which we studied previously for Ig gene rearrangements (4), was retrospectively found to express granzyme B and TIA-1 in a fraction of H/RS cells (Table I). 20 $CD30^+$ H/RS cells and 10 TIA-1^+ H/RS cells were micromanipulated and analyzed for IgH and Igκ gene rearrangements and configuration of the TCR-β loci. Two clonal IgH and one clonal Igκ gene rearrangement were amplified from H/RS cells regardless of their phenotype (Tables II and III). V_H and Vκ genes were somatically mutated. The Vκ region gene and one of the V_H gene rearrangements, which had both likely been originally productive, were rendered nonfunctional by somatic mutations (Table III). For unknown reasons, a second clonal Vκ gene rearrangement amplified in the first analysis was not obtained in the present study. No TCR-β gene rearrangements, but instead germline configuration for the TCR Cβ1 and Cβ2 loci, were detected (Table II).

Taking cases I and II together, amplification of clonal V_H gene rearrangements rendered non-functional by deleterious somatic mutations together with the detection of germline configuration in both TCR-β loci identify the H/RS cells in these cases as the progeny of germinal center B cells that have lost their capacity to express antigen receptor due to "crippling" somatic mutations.

From case III, in which virtually all H/RS cell express perforin (Table I), 30 $CD30^+$ H/RS cells were analyzed for IgH, Igκ, and Igλ gene rearrangements (half of the cells were in addition analyzed with V_H leader, IgH DJ, and IgH germline primer collections). Only one IgH VDJ gene amplificate was obtained, likely representing cellular or other contamination (Table II). No IgH DJ gene rearrangement was obtained. However, a fragment specific for germline configuration was repeatedly amplified from H/RS cells. Within these IgH germline fragments, two distinct sequences of the $J_H\psi1$ pseudogene (G and/or A at position 45 of the $J_H\psi1$ pseudogene; see reference 16) were detected, suggesting that both alleles of the IgH locus are in germline configuration. In contrast, analysis of the TCR-β loci yielded two clonal rearrangements involving the two alleles of the TCR Cβ1 locus in about half of the H/RS cells. One allele harbors a clonal Vβ7.1–Dβ1–Jβ1.6 rearrangement (Fig. 2 C), whereas the other carries a clonal Dβ1–Jβ1.4 gene rearrangement (Fig. 2 D). The Vβ7.1 gene rearrangement is potentially functional. Unexpectedly, it exhibits intraclonal diversity (Fig. 2 C). Three different sequences were obtained (Fig. 2 C, sequences A–C). The three sequence variants were confirmed by repeated reamplification and sequencing from distinct aliquots of the whole genome amplification. Taken together, in case III the single cell PCR results identify a T cell as the progenitor of the tumor clone, thus classifying this case of cHD as a T cell lymphoma.

Discussion

Derivation of H/RS cells from mature B cells was previously demonstrated in 18 out of 18 informative unselected cases of cHD analyzed in Cologne and Frankfurt (3–5, 17–19). In addition, recent results obtained by others indicate B cell derivation of H/RS cells in 24 out of 25 cases of cHD (20). On the other hand, in a nonselected collection of 13 cases of primary cHD, Daus and colleagues did not detect clonal TCR-γ gene rearrangements in micromanipulated H/RS cells from any of these cases (21). Therefore, these data collectively indicate that cHD represents a homogenous entity as a B cell lymphoma.

Table II. *Summary of Single Cell PCR Analysis of Three Cases of cHD*

Case	Gene locus*	Staining for micromanipulation	H/RS cells positive	PCR products		Rearrangements or *germline configuration*		Buffer controls positive
				Total	Sequenced	Repeated	Unique‡	
I	IgH	CD30	21/50	19 V_H2	17	17 V_H2		0/15
				1 V_H3	1		1 V_H3	
				1 V_H4	1		1 V_H4	
			9/14	9 D_H3–J_H	9	9 D_H3-9–J_H6c		0/4
		Granzyme B	3/15	3 V_H2	3	3 V_H2		
	Igκ	CD30	0/16					0/4
	Igλ	CD30	0/8					0/4
	TCR Cβ1	CD30	9/20	9 germline	9	9 *germline*		0/14
		Granzyme B	3/15	3 germline	3	3 *germline*		
	TCR Cβ2	CD30	9/20	9 germline	9	9 *germline*§		0/14
		Granzyme B	3/15	3 germline	2	2 *germline*		
	EBNA1	CD30	7/10	7 EBNA1				0/4
II	IgH	CD30	8/20	6 V_H1	6	6 V_H1		0/6
				7 V_H3	6	6 V_H3		
		TIA-1	3/10	2 V_H1	2	2 V_H1		0/6
				3 V_H3	2	2 V_H3		
	Igκ	CD30	9/20	9 Vκ1	7	7 Vκ1		0/6
		TIA-1	2/10	3 Vκ1	2	2 Vκ1		
	TCR Cβ1	CD30	4/20	4 germline				0/4
		TIA-1	6/10	6 germline				
	TCR Cβ2	CD30	9/20	9 germline				0/4
		TIA-1	4/10	4 germline				
	EBNA1	CD30	8/20	8 EBNA1				0/6
		TIA-1	5/10	5 EBNA1				
III	IgH	CD30	1/30‖	1 V_H3	1		1 V_H3	0/10
			6/15	6 germline	6	6 *germline*§		0/4
	Igκ	CD30	0/30					0/10
	Igλ	CD30	0/30					0/10
	TCR Cβ1	CD30	20/30	14 Vβ7	14	14 Vβ7		1/10¶
				14 Dβ1–Jβ1	14	14 Dβ1–Jβ1.4		
				1 germline	1		1 *germline*	
	TCR Cβ2	CD30	17/30	17 germline	7	7 *germline*		0/10
	EBNA1	CD30	0/10					0/4

Controls:			Micromanipulated		Sorted by flow cytometry	
	Gene locus		B cells	T cells	B cells	T cells
	IgH	VDJ	5/22	0/12	8/15	
		DJ/germline			9/10	
	Igκ		3/14		4/7	
	Igλ		3/14		3/7	
	TCR-β	VDJ		6/22		7/9
		DJ/germline	16/22		16/18	

*For IgH and IgL loci, only V(D)J rearrangements; for TCR-β loci, VDJ and DJ rearrangements as well as fragments specific for germline configurations were amplified. Cases I and III were also analyzed for D_HJ_H rearrangements and germline configuration of the IgH locus.
‡Representing most likely cellular or other contamination.
§These loci were found in germline configuration on both alleles due to a germline polymorphism of the Dβ2 gene (case I) and the JHψ1 gene (case III).
‖15 of the H/RS cells from case III were in addition analyzed using V_H leader primers.
¶The contamination amplifed from one buffer control represents a TCR Cβ1 germline PCR product.

Table III. *Sequence Analysis of Clonal Ig and TCR-β Gene Rearrangements*

Case	Gene locus	Rearrangement*	Potentially functional	Percent mutation	Remarks
I	IgH	V_H2-5–D_H3-10–J_H5b	No‡	4.3	Stop (codon 91)
		D_H3-9–J_H6c	n.a.	0.9	
					TCR-β loci in germline
II	IgH	V_H1-8–D_H3-22–J_H6b§	No‖	3.5	47-bp deletion;
	IgH	V_H3-53–D_H2-2–J_H6b§	No¶	1.9	Stop in CDR3
	Igκ§	Vκ1 (L12)–Jκ1§	No‡	1.3	Stop in CDR3
					TCR-β loci in germline
III	TCR-β	Vβ7.1–Dβ1–Jβ1.6	Yes	0.3–0.7	Sequence variations involving codons 98, 105, and 108; three variants
	TCR-β	Dβ1–Jβ1.4	n.a.	0	
					IgH locus in germline

n.a., not applicable.
*Sequences of cases I and III are available from GenBank/EMBL/DDBJ under accession nos. AJ243643–AJ243648 and AJ251000–AJ251002.
‡These rearrangements were potentially functional before introduction of a stop codon.
§Sequences of this case have been published previously (reference 4).
‖This rearrangement was potentially functional before the 47-bp deletion.
¶This rearrangement was likely originally nonfunctional due to a stop codon generated during VDJ recombination.

However, there are some observations raising the possibility that—in a subset of cHD—the tumor cells might stem from T lymphocytes. Some putative H/RS cell lines, for example, are derived from T cells (2). For these cell lines, proof of derivation from H/RS cells in the patients is missing. Furthermore, a TCR-α gene rearrangement was amplified from whole tissue DNA of lymphomatoid papulosis, cHD, and anaplastic large cell lymphoma occurring sequentially in one individual patient, suggesting a common T cell derivation of the three diseases (22). However, assignment of T cell genotype to H/RS cells of the Hodgkin's lymphoma was not conclusive: anaplastic large cell lymphoma versus classic cHD is often a difficult differential diagnosis (1, 14). In this case, a clear discrimination between the two entities was particularly complicated because H/RS cells coexpressing CD30 and CD15 were found in both lymphomas. Furthermore, the TCR-α gene rearrangement identified in the Hodgkin's disease biopsy with the help of clone-specific primers represented a faint band, which might have arisen from a few contaminating cells originating from either the lymphomatoid papulosis or the anaplastic large cell lymphoma instead of the cHD. Finally, H/RS cells in a minority of cHD cases express cytotoxic granular molecules, in particular granzyme B, perforin, and TIA-1, which are otherwise typically found in cytotoxic T lymphocytes (6–9). Three such cases were analyzed here.

In cases I and II, molecular analysis of Ig and TCR-β loci revealed that the H/RS cells, despite expression of granzyme B and TIA-1 (Table I), were derived from germinal center B cells. Thus, expression of granzyme B and TIA-1 does not necessarily reflect a T cell origin of H/RS cells and shows that granzyme B and TIA-1 are aberrantly expressed by B lineage–derived H/RS cells. Similarly, expression of molecules thought to be specific for cells of the dendritic/myeloid lineage (23) by H/RS cells does not apparently reflect a derivation of these cells from dendritic or myeloid cells. Thus, H/RS cells can mimic cells of various hematopoietic lineages in terms of cell surface marker expression.

In case III, the H/RS cells harbor a clonal TCR-β VDJ and a clonal DJ gene rearrangement but no clonal Ig gene rearrangement. Furthermore, the IgH locus was found in germline configuration biallelically, directly demonstrating the absence of clonal IgH gene rearrangements. Given that the presence of a TCR-β VDJ gene rearrangement defines a T cell, the H/RS cells in this case are derived from a T lymphocyte. The Vβ7.1 gene rearrangement amplified from the T cell tumor clone exhibits significant intraclonal diversity. This was not expected, since TCR genes in T cells are usually not subject to somatic hypermutation, although there are some reports claiming the rare occurrence of somatically mutated TCR genes (24–26). Notably, somatic mutations were not observed in the Dβ1–Jβ1.4 rearrangement or in intronic sequences flanking the germline Dβ2 gene (830-bp sequences; see Table II). The distribution of somatic mutations among the distinct gene fragments of the TCR-β loci argues in favor of somatic hypermutation rather than some other type of somatic mutation (e.g., "genomic instability") as the cause for the mutations in the TCR-β VDJ genes: in analogy to the Ig loci, somatic hypermutation would be expected to preferentially target VDJ joints rather than DJ rearrangements or germline genes, whereas genomic instability should not specifically target rearranged VDJ genes. Whether these mutations indeed reflect somatic hypermutation outside Ig loci, however, remains unclear.

In ~5–15% of all cases of cHD, the H/RS cells exhibit cytotoxic T cell phenotype. Studying three of these cases, we

found one to be T cell derived. Apart from this, 18 out of 18 nonselected cases of cHD in our collection are B lineage derived. On this basis, a rough estimate would be that cHD occurs as a T cell lymphoma at a low frequency (i.e., <5%).

This study establishes that rare cases of cHD derived from T cells indeed exist, indicating that cHD as defined by histopathology is not a uniform disease. It is remarkable that the transformation of both T and B cells can lead to the H/RS cell phenotype. Whether this reflects an initial transforming event inside the germinal center microenvironment in both cases and whether T and B cell derived H/RS cells can be distinguished in terms of gene expression patterns remain to be established.

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